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14. ABSTRACT

Two studies related to breast cancer prevention were conducted: 1) The effects of treatment of femaile Sprague-Dawley rats with indole-3-carbinol (I3C), tamoxifen (TAM) or TAM+I3C on caspase activities in the mammary gland and mammary tumors induced with 7,12-dimethylbenz[α]anthracene (DMBA). Activities of caspase-3+7,-6 and -9 in the mammary gland of tumor-free DMBA-initiated rats were up-regulated by a 14 to 58 treatments with I3C. DMBA initiation was found to reduce the activities of caspase-3+7 and -8 in the mammary gland. 2) The effects of treatment of rats with 3,3'-diindolylmethane (DIM), the dimmer of I3C formed in gastric acid, on the gene expression level of hepatic cytochrome P450s (CYPs) involved in estrogen metabolism. The results indicated that treatment with Phenobarbital (PB), a relevant inducer, increased mRNA expression of CYP2B1/2 significantly and 3A1.2 insignificantly, and treatment with DIM insignificantly up-regulated hepatic mRNA levels of CYP1A1 and 2B1/2. When combined with PB, treatment with DIM significantly induced mRNA level of CYP1A1, and at low does level of DIM, CYP2B1, and 3A2. These two studies suggested the prevention of mammary tumorigenesis by I3C and DIM may be explained by their effects on apoptosis and estrogen metabolism.

15. SUBJECT TERMS
Breast cancer, chemoprevention, indole-3-carbinol, tamoxifen, 3,3'-diindolylmethane, cytochrome P450, apoptosis, caspase

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Introduction

Indole-3-carbinol (**I3C**) is a product of autolysis from glucobrassicin in cruciferous vegetables. It is condensed to 3,3'-diindolylmethane (**DIM**) and other products in gastric acid. I3C and DIM have been found effective in suppression of breast cancer *in vivo* and *in vitro* (Bradlow *et al.*, 1991; Chen *et al.*, 1998; Firestone *et al.*, 2003).

Previous studies from this laboratory showed that caspase activities in the mammary gland were increased by short-term treatment of rats with I3C (Zhang and Malejka-Giganti, 2003). It might have been due to the promotion by I3C of Bax translocation to mitochondria to induce apoptosis (Sarkar *et al.*, 2003). Tamoxifen (TAM) has also been reported to induce caspase activities preceding apoptosis in rat mammary tumors *in vivo* as well as in both estrogen receptor-negative and -positive human breast cancer cells *in vitro* (Mandlekar et al., 2000). Therefore, the preventive effects on breast cancer by I3C may be ascribed to its activation of caspase cascade from caspase-8 (**C8**) and/or caspase-9 (**C9**) to downstream effector caspase-3 (**C3**), caspase-6 (**C6**) and caspase-7 (**C7**). Hence, I3C in an adjuvant therapy with TAM may cooperatively or synergistically inhibit tumorigenesis by inducing apoptosis in mammary glands and tumors. Therefore, caspase activities in the mammary gland and 7,12-dimethylbenz[α]anthracene (DMBA)-initiated mammary tumors were examined upon treatment of rats with I3C and/or TAM.

Modulations of hepatic phase I and phase II enzymes in detoxification of carcinogens such as estradiol may be another mechanism of anti-mammary tumorigenesis by I3C or DIM (Bradlow *et al.*, 1991; Wortelboer *et al.*, 1992). Whether DIM alters the expression of phase I enzymes at gene level is still unclear. In this study, hepatic mRNA levels of cytochrome P-450 (CYP) were assayed after treatment of rat with DIM and/or phenobarbital (PB), which is an inducer of hepatic CYPs involved in metabolism of estrogens.

Body

Study design

1. Two weeks after one oral dose of 65 mg/kg bw of DMBA, 9-wk-old female Sprague-Dawley rats were divided into four groups and treated 3 times per week, up to 58 times, with: **(1)** Vehicle I of TAM (Veh I, 10% ethanol in olive oil at 0.1 ml/rat, s.c.) + Vehicle II of I3C (Veh II, 20% ethanol in olive oil at 2.5 ml/kg bw, i.g.), **(2)** TAM (10 μg per rat in 50 μl Veh I, s.c.) + Veh II, **(3)** Veh I + I3C (250 mg/kg in Veh II, i.g.) and **(4)** TAM + I3C as above. Control (non-DMBA) rats also consisted of four groups treated as above. The rats were sacrificed when tumors reached volume of about 1 cc. The control and non-tumor bearing DMBA-initiated rats from each treatment group were also sacrificed at the corresponding time intervals. The mammary glands and tumors were isolated for histological evaluation and protein extraction. Caspase activities of C3+7, C6, C8 and C9 were measured using modified colorimetric caspase assay system (Promega, USA). The mean natural logarithms of activity ± standard deviation were analyzed using a one-way analysis of variance (SPSS, USA) to compare the effects of different treatment regimens after a short-term (a total of 8 treatments within 2.6 weeks) or a long-term (from 14 to 58 treatments during ~ 20 weeks) administration. The significance level was set at P=0.05.

2. Seven-wk-old female SD rats were treated with either DIM (8.4 or 42 mg/kg b.wt.) or vehicle (20% ethanol in olive oil) by oral *gavage* and either PB (75 mg/kg b.wt.) or saline by i.p. injection (2.5 ml/kg b.wt) once daily for 4 consecutive days. Rats were terminated 24 hr after the last treatment and a small portion of liver was snap frozen in liquid for total RNA isolation using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized by pre-incubating 5 μg of total RNA, 1.25 μg of oligo$(dT)_{15}$ primers, 25 nmole of each dNTP at 65°C for 5 min followed by incubation at 50°C for 1 hr with 5 mM DTT, 500 U of Superscript III reverse transcriptase and 10 μl of 5X reaction buffer (Invitrogen), in a total volume of 50 μl. The cDNA levels of CYPs were detected by real-time PCR with specific primer pairs that flank in each side of intron-extron junctions and amplify fragments between 100bp and 150 bp in length. The 25 μl PCR reaction containing 12.5 μl of Platinum SYBR Green qPCR SuperMix-UDG, 0.5 μl of ROX reference dye (Invitrogen), 150 nM each primer and 1 μl of reverse transcription reaction mixture was programmed as 1 cycle at 50°C for 2 min and 95°C for 2 min, 50 cycles at 95°C for 15 sec and 60°C for 30 sec. The CYP mRNA level was expressed relative to 1,000 copies of beta-actin mRNA, the internal control.

Results

1. Effects of treatment of rats with I3C and/or TAM on caspase activities in the mammary gland and malignant mammary tumor (Fig. 1)

After a short-term treatment (8 treatments within 2.6 weeks), the level of C3+7 activity was greater (P=0.072) and the level of C8 activity was significantly greater (P=0.004) in the mammary gland (MG) of non-DMBA rats than those in the mammary gland of tumor-free [MG(t-)] DMBA-initiated rats, irrespective of treatment regimen.

In the mammary gland of tumor-free [MT(t-)] DMBA-initiated rats, the levels of all caspase activities were higher in I3C-treated than vehicle-treated group; and those in TAM+I3C-treated group were higher than those of TAM-treated one but lower than those of I3C-treated group. In the mammary gland (MG) of non-DMBA rats, the levels of all caspase activities were the highest in TAM+I3C-treated group. None of those differences was significant.

During a long-term treatment (from 14 through 58 treatments), the levels of all caspase activities were significantly greater in mammary tumor than those in the mammary gland of rats with or without DMBA-initiation (P<0.001). The levels of C3+7 and C8 activities were significantly greater in the mammary gland of non-DMBA rats than those in both MG(t-) and MG(t+) of DMBA-initiated rats (P<0.001). The level of C8 activity was significantly greater (P<0.001) in the mammary gland of tumor-bearing [MG(t+)] than tumor-free [MG(t-)] DMBA-initiated rats and the level of C9 activity was in the opposite way (P=0.038).

In the mammary gland of tumor-free [MG(t-)] DMBA-initiated rats, the level of C3+7 activity was significantly higher in I3C-treated group (P=0.024) and higher in TAM+I3C-treated group (P=0.065) than that in vehicle-treated group; the level of C6 activity was higher (P=0.06) in I3C-treated group than that in vehicle-treated group; the level of C9 activity in I3C-treated group was significantly higher than that in vehicle-treated group (P=0.01) and higher than that in TAM-treated group (P=0.075). However, compared to treatment with vehicle, those with I3C and/or TAM did not significantly change the levels of caspase activity in mammary tumors (MT), mammary gland (MG) of non-DMBA rats or tumor-bearing [MG(t+)] DMBA-initiated rats.

The treatment phase or rat age associated with the levels of caspase activities. Generally, the number of treatment with TAM effected the levels of C3+7 and C9 activity (P=0.041 and 0.052, respectively) and the number of treatment with I3C effected the levels of C3+7, C6 and C9 activity (P=0.047, 0.044 and 0.022, respectively). Such affects were considered in all the above comparisons.

2. Effects of a short-term treatment of rats with PB and/or DIM on the hepatic CYP mRNA expression (Fig. 2)

Treatment with PB up-regulated the hepatic mRNA level of CYP2B1 (P=0.055) and significantly of 2B2 (P=0.004). It insignificantly up-regulated the expression of CYP3A1 and 3A2, but when combined with lower dose level and both dose levels of DIM, treatment with PB significantly up-regulated the mRNA levels of CYP3A1 and 3A2, respectively. Treatment with PB possibly up-regulated CYP1A1 transcription only when it was combined with higher dose level of DIM. Treatment with PB alone significantly down-regulated the mRNA level of CYP2E1 in liver (P=0.03, data not shown).

Treatment with DIM up-regulated the hepatic mRNA levels of CYP1A1, 2B1 and 2B2. The induction was greater at higher dose level of DIM than that at the lower dose level, but none of them were significant. However, when combined with PB, treatment with both dose levels of DIM significantly induced mRNA level of CYP1A1 and with lower dose level of DIM significantly induced those of CYP2B1 and CYP3A2. Treatment with DIM alone insignificantly down-regulated hepatic mRNA levels of CYP3A1/2 and apparently decreased CYP2E1 mRNA level (data not shown).

Key Research Accomplishments

- Initiation of mammary tumorigenesis with DMBA suppressed caspase-8 and slightly later (~3 weeks after initiation) caspase-3&7 activities in the mammary gland.
- Treatment of rats with I3C increased caspase activities in the mammary gland, especially the caspase-3&7 and caspase-8 activities in mammary gland of tumor-free DMBA-initiated rats in a long-term treatment.
- Treatment of rats with PB induced hepatic mRNA levels of 2B1/2 and 3A1/2 and reduced the level of CYP2E1.
- Treatment with DIM induced hepatic mRNA levels of CYP1A1 especially when combined with PB, and when combined with PB, it induced CYP2B1 and 3A2 at low dose level.

Reportable Outcomes

Caspase activities: Manuscript in preparation, intended submission to Cancer Epidemiology, Biomarkers and Prevention.

CYP mRNA expression:

1. Poster at the 96th AACR Annual Meeting, Anaheim, CA, April 16-20, 2005 (Appendices: Abstract)
2. Poster at the Engebretson Symposium on Drug Discovery and Development In Cancer Experimental Therapeutics, Minneapolis, MN, June 9, 2005
3. Poster at the VA Medical Center Research Day, Minneapolis, MN, June 24, 2005

Conclusions

The levels of caspase activities in the mammary tumor were much higher than those in the mammary gland and were not affected by treatment of rats with TAM and/or I3C.

Initiation of rats with DMBA significantly decreased the activities of C8 in mammary gland and later, about three weeks after initiation, decreased the activities of C3+7, suggesting that the mechanism of tumorigenesis by DMBA may depend on inhibition of extrinsic pathway of apoptosis, i.e., DMBA suppresses the activation of C8 and subsequently the activities of effector C3+7, saving abnormal cells from apoptosis and offering them more chances to survive and propagate. Comparing the levels of caspase activities in mammary gland of tumor-bearing rats to those of tumor-free DMBA-initiated ones, C8 activity was greater in the former [MG(t+)] and C9 greater in the latter [MG(t-)], indicating that the existence of mammary tumor increased the level of C8 activity and decreased that of C9 in other parts of mammary gland. It is a possible systemic response to tumor by the body, or tumor itself suppresses tumorigenesis in other parts of the body through activating transmembrane death receptors and subsequently the extrinsic pathway of apoptosis.

In the mammary gland of tumor-free but not tumor-bearing DMBA-initiated rats, treatment with I3C increased caspase activities, and even to a greater extent after a long-term treatment, implying that I3C may be protective in suppressing mammary tumorigenesis by increasing apoptosis before tumors have fully developed. However, no additive or synergistic effects on caspase activities were observed by treatment with TAM+I3C. It suggests a prophylactic value of I3C in mammary tumor prevention.

A short-term treatment of rats with PB preferentially up-regulated mRNA expression of hepatic CYP2B1 and CYP2B2 and to a lesser extent of CYP3A1/2. Treatment with DIM insignificantly up-regulated mRNA levels of hepatic CYP1A1 and 2B1/2 and decreased those of CYP3A1/2. The treatment of rat with PB+DIM increased hepatic mRNA expression of CYP1A1 due to the effect of DIM, and increased the expression of CYP2B1/2 and 3A1/2 basically due to PB. Therefore, treatment with DIM has moderate effects on the expression levels of phase I enzymes and may subsequently change the metabolism of estrogens.

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Appendices

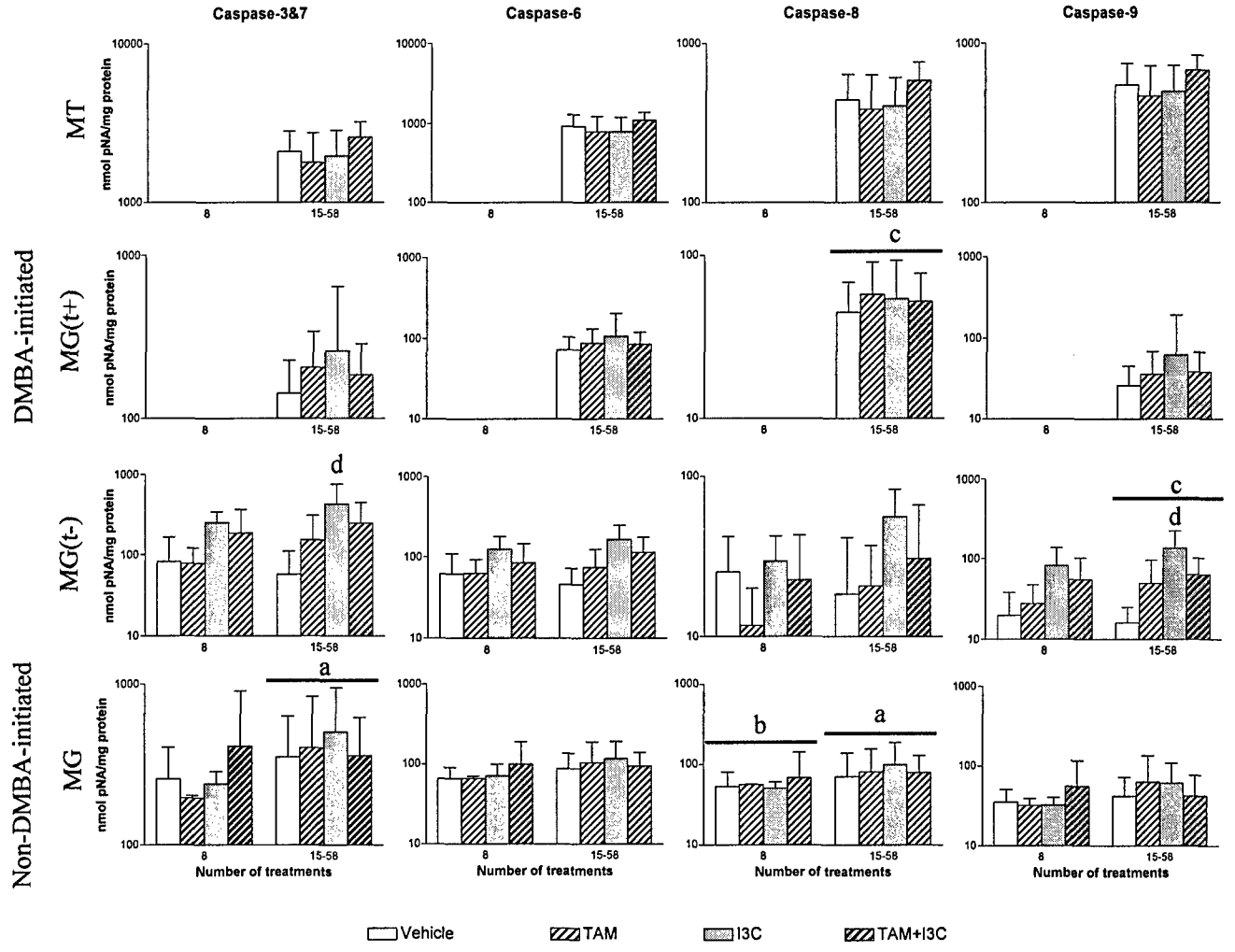

Fig. 1. Caspase activities (arithmetic means ± SD) in mammary gland (MG) of non-DMBA rats and mammary gland with tumor [MG(t+)] or without tumor [MG(t+)] and malignant mammary tumors (MT) of DMBA-initiated rats after a short-term or long-term treatment with I3C and/or TAM. The data are presented as the quantity of p-nitroaniline (nmol) liberated per mg protein per 24 hr. a, $P < 0.05$ vs. corresponding long-term treatment groups of MG(t-) and MG(t+) of DMBA-initiated rats; b, $P < 0.05$ vs. corresponding short-term treatment groups of MG(t-); c, $P < 0.05$ vs. corresponding tumor-bearing or -free groups; d, $P < 0.05$ vs. corresponding vehicle-treated group.

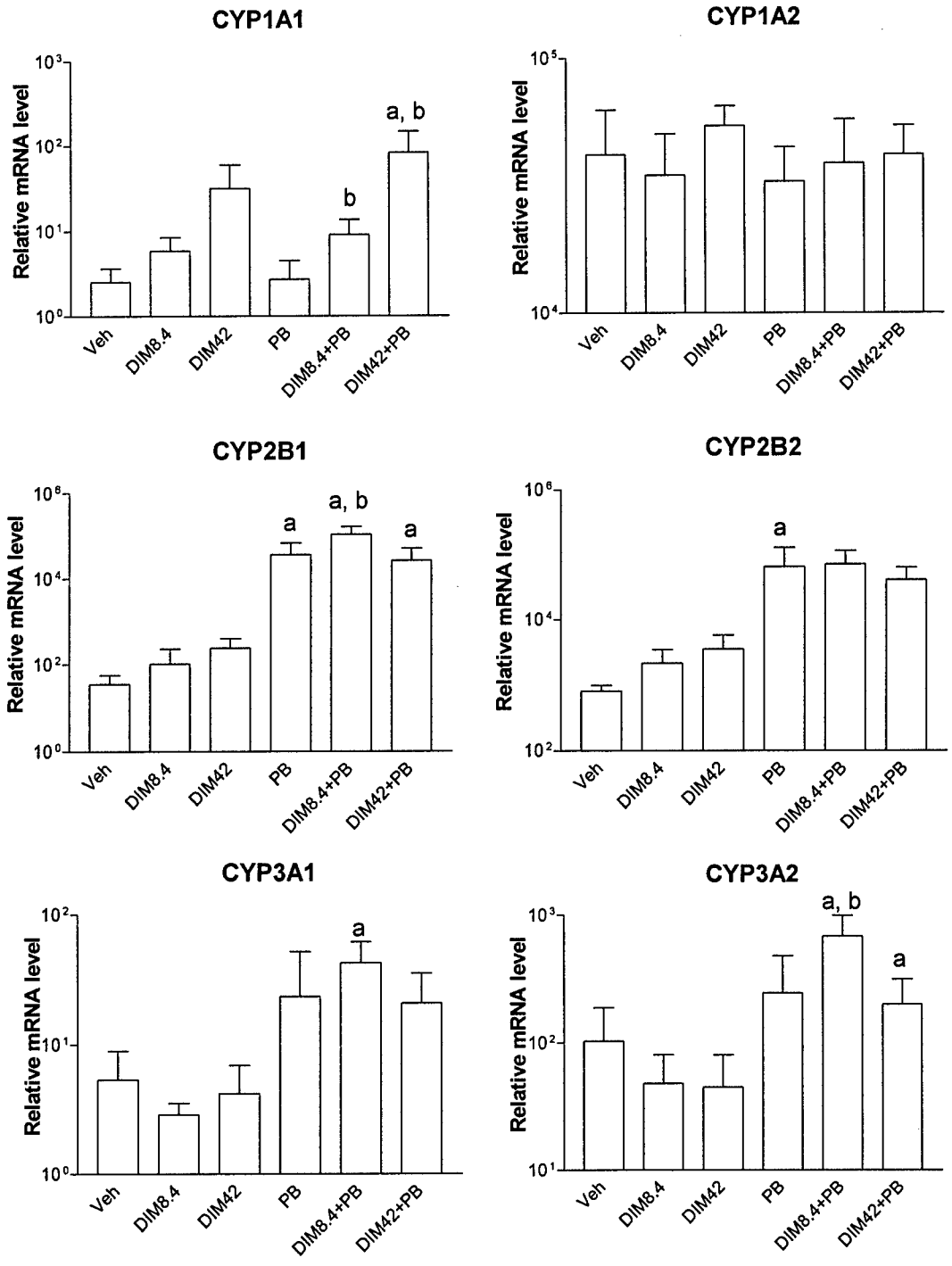

Fig. 2. Effects of treatment of rats with DIM, PB or DIM+PB on CYP mRNA expression in the liver. The data are presented as the mean ± SD. a, $P < 0.05$ vs. corresponding non PB-treated group; b, $P < 0.05$ vs. PB-treated group.

Abstract for 96th AACR Annual Meeting, Anaheim, CA, April 16-20, 2005

Inhibition by 3,3′-diindolylmethane *in vivo* of 2-, 4- and 6β-hydroxylations of 17β-estradiol by rat hepatic microsomes: a potential mechanism for suppression of mammary cancer

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Cancer-preventive properties of the components of cruciferous vegetables including indoles have been studied extensively. 3,3′-Diindolylmethane (DIM) is a major product formed in the acid pH of the stomach after oral intake of indole-3-carbinol. DIM has been reported to inhibit activities of CYP1A1 and CYP2B1/2 *in vitro* (Stresser *et al*, *J Biochem Toxicol*, 1995). Since phenobarbital (PB)-inducible CYP2B1/2 catalyzes 4-, 6α- and 6β-hydroxylations of 17β-estradiol (E2) yielding putative carcinogenic metabolites of E2, we investigated whether treatment of female Sprague-Dawley rats with DIM affects the PB-inducible CYP mRNA expression, CYP activities and metabolism of E2. At 50 days of age, rats were treated with PB (75 mg/kg b.wt. by intraperitoneal injection), DIM (42 mg/kg b.wt. by oral gavage), PB+DIM or the respective vehicles for four consecutive days. On day 5, rats were killed and the livers were excised and processed for analyses of CYP (1A1, 1A2, 2B1, 2B2, 3A1 and 3A2) mRNA expression by real-time PCR, CYP activities by *O*-dealkylation of alkoxyresorufins and nifedipine oxidation (NIFOX), and [4-^{14}C]-E2 metabolism by HPLC and radiometry. Treatment of rats with DIM increased the relative mRNA expression level of CYP1A1 (9-fold), CYP2B1 (6-fold) and CYP2B2 (4-fold). Treatment with PB preferentially induced mRNA expression of CYP2B1 (505-fold) and CYP2B2 (34-fold), and increased that of CYP3A2 (2-fold). Treatment with DIM increased EROD (CYP1A1), BROD and PROD (CYP2B1/2) activities 8-, 27- and 9-fold, respectively. Treatment with PB increased EROD, BROD, PROD and NIFOX (CYP3A1/2) activities (2.2-, 725-, 311- and 1.5-fold, respectively) and the total P450 level (1.8-fold). Neither DIM nor PB treatment affected MROD (CYP1A2) activity. Treatment with DIM increased the rates of formation of 2-OH-E2 (1.5-fold) and 6β-OH-E2 (2.2-fold) from E2, while PB increased the rates of formation of 2-OH-E2, 2-OH-E1, 4-OH-E2, E3, 6α-OH-E2, 6β-OH-E2 and 6(α+β)-OH-E1 (3.6-, 16-, 3.1-, 4.4-, 5.4-, 32- and 2.5-fold, respectively) from E2. Treatment with DIM+PB decreased the rates of formation of 2-OH-E2, 2-OH-E1, 4-OH-E2, 6β-OH-E2 and 6(α+β)-OH-E1 from E2 by 28, 63, 43, 47 and 49%, respectively, relative to PB-treated rats, indicating partial inhibition by DIM *in vivo* of the PB-induced increases in the rates of oxidation of E2. This was not associated with down-regulation of either the mRNA expression or activity of the CYPs that reportedly catalyze these hydroxylations. These findings suggest that DIM might inhibit a CYP not analyzed in this study or that DIM might be a substrate for E2-metabolizing CYP(s), and thus decreases its capacity to hydroxylate E2. The mechanism of inhibition by DIM *in vivo* of E2 oxidations deserves further investigation since E2 plays an important role in the promotion of mammary carcinogenesis. Inhibition by DIM of adverse E2 reactions, particularly 4-, 6α- and 6β-hydroxylations, might offer the desired means of suppressing mammary cancer.

[This research was supported by the Department of Veterans Affairs and the Department of Defense.]